PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K

A2

(11) International Publication Number: WO 92/20316

(43) International Publication Date: 26 November 1992 (26.11.92)

(21) International Application Number: PCT/US92/03875

(22) International Filing Date: 14 May 1992 (14.05.92)

(30) Priority data:
699,891 14 May 1991 (14.05.91) US

(71) Applicants: UNIVERSITY OF CONNECTICUT [US/US]; Storrs, CT 06269 (US). TARGETECH, INC. [US/US]; 290 Pratt Street, Meriden, CT 06450 (US).

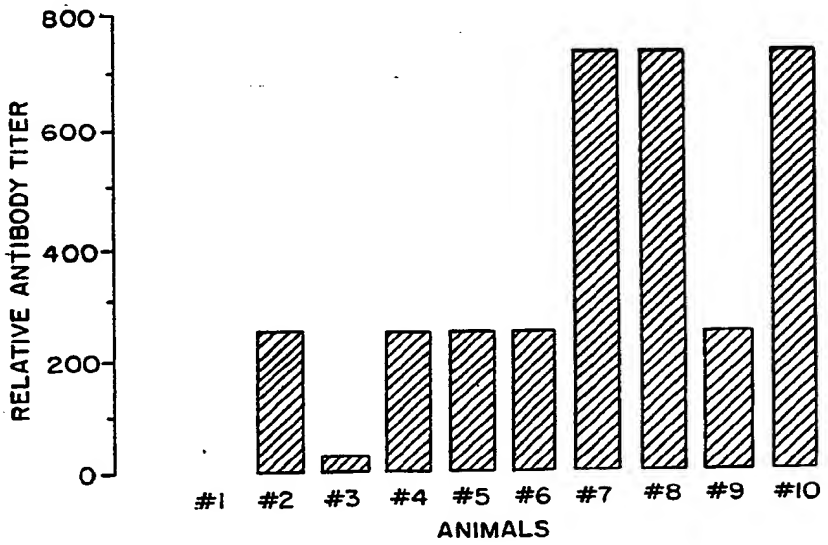
(72) Inventors: WU, George, Y. ; 15 Rundelane, Bloomfield, CT 06002 (US). SPITALNY, George, L. ; 6 Brookfield Court, Cheshire, CT 06410 (US). FINDEIS, Mark, A. ; 234 Sherman Avenue, Apt. #29, Meriden, CT 06450 (US).

(74) Agents: DECONTI, Giulio, A., Jr. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: TARGETED DELIVERY OF GENES ENCODING IMMUNOGENIC PROTEINS



(57) Abstract

Molecular complexes can be used to target a gene encoding an immunogenic protein or polypeptide to a specific cell *in vivo*. The gene is incorporated into the target cell, expressed and the gene-encoded product is secreted and an immune response against the immunogenic protein or polypeptide is elicited. The molecular complex comprises an expressible gene encoding a desired immunogenic protein or polypeptide complexed with a carrier of a cell-specific binding agent and a gene-binding agent. The cell-specific binding agent is specific for a cellular surface structure which mediates internalization of ligands by endocytosis. An example is the asialoglycoprotein receptor of hepatocytes. The gene-binding agent is a compound such as a polycation which stably complexes the gene under extracellular conditions and releases the gene under intracellular conditions so that it can function within a cell. The molecular complex is stable and soluble in physiological fluids and can be used to elicit an immune response against a variety of immunogens in an organism for the purpose of vaccination or for the production of antibodies.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CS	Czechoslovakia
DE	Germany
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
MC	Monaco
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
NL	Netherlands
NO	Norway
PL	Poland
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
US	United States of America

TARGETED DELIVERY OF GENES ENCODING IMMUNOGENIC PROTEINS

Background of the Invention

Immunization of animals for the purpose of vaccination or production of antibodies (used for passive immunization, diagnostic or scientific reagents) typically involves injection of a natural or recombinant protein which has been partially or completely purified to homogeneity. Purification of the protein to homogeneity usually requires several steps involving anionic, cationic and molecular sieve chromatography. These procedures are time consuming and the homogeneity of the final product must be verified. Alternatively, a differentiating assay which can distinguish the protein of choice from all other proteins can be used to develop a monoclonal antibody.

Summary of the Invention

This invention pertains to a soluble molecular complex for targeting, a gene (or genes) encoding an immunogenic protein or polypeptide for which an immune response is desired, to a specific cell in a host organism in vivo and obtaining expression of the gene, production of the gene-encoded protein or polypeptide and development of an immune response against the immunogenic protein or polypeptide in the host organism. The molecular complex comprises a

nucleic acid molecule containing an expressible gene encoding a desired immunogenic protein or polypeptide complexed with a carrier which is a conjugate of a cell-specific binding agent and a gene-binding agent. The cell-specific binding agent is specific for a cellular surface structure, typically a receptor, which mediates internalization of bound ligands by endocytosis, such as the asialoglycoprotein receptor of hepatocytes. The cell-specific binding agent can be a natural or synthetic ligand (for example, a protein, polypeptide, glycoprotein, etc.) or it can be an antibody, or an analogue thereof, which specifically binds a cellular surface structure which then mediates internalization of the bound complex. The gene-binding component of the conjugate is a compound such as a polycation which stably complexes the gene under extracellular conditions and releases the gene under intracellular conditions so that it can function within the cell.

The complex of the gene and the carrier is stable and soluble in physiological fluids. It can be administered in vivo where it is selectively taken up by the target cell via the surface-structure-mediated endocytotic pathway. The incorporated gene is expressed, the gene-encoded protein or polypeptide is processed and secreted as a soluble or a cell surface protein or polypeptide by the transfected cell and an immune response is evoked against the protein or polypeptide in the host organism.

The soluble molecular complex of this invention can be used to elicit an immune response in an organism to a desired immunogenic protein or

polypeptide. It can be used for immunization of organisms for the purpose of vaccination or for the production of antibodies for experimental (e.g., research reagent), diagnostic or therapeutic use.

Brief Description of the Figure

Figure 1 is a bar graph representing the relative titer of anti-HBsAg antibody in animals receiving the molecular complex of the invention.

Detailed Description of the Invention

A soluble, targetable molecular complex is used to deliver a gene encoding an immunogenic protein or polypeptide to a target cell or tissue in vivo and obtain expression of the gene, production of the gene-encoded protein or polypeptide and development of an immune response against the immunogenic protein or polypeptide in a host organism. The molecular complex comprises the gene encoding a desired immunogenic protein or polypeptide to be delivered complexed with a carrier made up of a binding agent specific for the target cell and a gene-binding agent.

The gene, generally in the form of DNA, encodes the desired immunogenic protein or polypeptide. Typically, the gene comprises a structural gene encoding the immunogenic protein or polypeptide in a form suitable for processing and secretion as a soluble or cell surface protein or polypeptide by the target cell. For example, the gene encodes appropriate signal sequences which direct processing and secretion of the protein or polypeptide. The signal sequence may be the natural sequence of the protein or exogenous sequences. The structural gene

is linked to appropriate genetic regulatory elements required for expression of the gene-encoded protein or polypeptide by the target cell. These include a promoter and optionally an enhancer element operable in the target cell. The gene can be contained in an expression vector such as a plasmid or a transposable genetic element along with the genetic regulatory elements necessary for expression of the gene and secretion of the gene-encoded product.

The carrier component of the complex is a conjugate of a cell-specific binding agent and a gene-binding agent. The cell-specific binding agent specifically binds a cellular surface structure which mediates internalization by, for example, the process of endocytosis. The surface structure can be a protein, polypeptide, carbohydrate, lipid or combination thereof. It is typically a surface receptor which mediates endocytosis of a ligand. Thus, the binding agent can be a natural or synthetic ligand which binds the receptor. The ligand can be a protein, polypeptide, glycoprotein or glycopeptide which has functional groups that are exposed sufficiently to be recognized by the cell surface structure. It can also be a component of a biological organism such as a virus, cells (e.g., mammalian, bacterial, protozoan) or artificial carriers such as liposomes.

The binding agent can also be an antibody, or an analogue of an antibody such as a single chain antibody which binds the cell surface structure.

Ligands useful in forming the carrier will vary according to the particular cell to be targeted. For targeting hepatocytes, glycoproteins having exposed

terminal carbohydrate groups such as asialoglycoprotein (galactose-terminal) can be used, although other ligands such as polypeptide hormones may also be employed. Examples of asialoglycoproteins include asialoorosomucoid, asialofetuin and desialylated vesicular stomatitis virus. Such ligands can be formed by chemical or enzymatic desialylation of glycoproteins that possess terminal sialic acid and penultimate galactose residues. Alternatively, asialoglycoprotein ligands can be formed by coupling galactose terminal carbohydrates such as lactose or arabinogalactan to non-galactose bearing proteins by reductive lactosamination.

For targeting the molecular complex to other cell surface receptors, other types of ligands can be used, such as mannose for macrophages, mannose-6-phosphate glycoproteins for fibroblasts, intrinsic factor-vitamin B12 for enterocytes and insulin for fat cells. Alternatively, the cell-specific binding agent can be a receptor or receptor-like molecule, such as an antibody which binds a ligand (e.g., internalizing antigen) on the cell surface. Such antibodies can be produced by standard procedures.

The gene-binding agent complexes the gene to be delivered. Complexation with the gene must be sufficiently stable in vivo to prevent significant uncoupling of the gene extracellularly prior to internalization by the target cell. However, the complex is cleavable under appropriate conditions within the cell so that the gene is released in functional form. For example, the complex can be labile in the acidic and enzyme rich environment of lysosomes. A noncovalent bond based on electrostatic

attraction between the gene-binding agent and the gene provides extracellular stability and is releasable under intracellular conditions.

Preferred gene-binding agents are polycations that bind negatively charged polynucleotides. These positively charged materials can bind noncovalently with the gene to form a soluble, targetable molecular complex which is stable extracellularly but releasable intracellularly. Suitable polycations are polylysine, polyarginine, polyornithine, basic proteins such as histones, avidin, protamines and the like. A preferred polycation is polylysine. Other noncovalent bonds that can be used to releasably link the expressible gene include hydrogen bonding, hydrophobic bonding, electrostatic bonding alone or in combination such as, anti-polynucleotide antibodies bound to polynucleotide, and strepavidin or avidin binding to polynucleotide containing biotinylated nucleotides.

The carrier can be formed by chemically linking the cell-specific binding agent and the gene-binding agent. The linkage is typically covalent. A preferred linkage is a peptide bond. This can be formed with a water soluble carbodiimide as described by Jung, G. et al. (1981) Biochem. Biophys. Res. Commun. 101:599-606. An alternative linkage is a disulfide bond.

The linkage reaction can be optimized for the particular cell-specific binding agent and gene-binding agent used to form the carrier. Reaction conditions can be designed to maximize linkage formation but to minimize the formation of aggregates of the carrier components. The optimal

ratio of cell-specific binding agent to gene-binding agent can be determined empirically. When polycations are used, the molar ratio of the components will vary with the size of the polycation and the size of the cell-specific binding agent. When a protein such as asialoorosomucoid and a polycation such as polylysine are used, the mass ratio of protein to polycation will typically be in the range of 5:1 to 1:5, preferably around 1:1. The molar ratios in these mixtures will vary widely with the molecular weights of the components. For the same protein, a greater number of molar equivalents of a lower molecular weight polycation will be required to prepare a conjugate with the same cation content as a conjugate prepared with a higher molecular weight polycation. Uncoupled components and aggregates can be separated from the carrier by molecular sieve chromatography or ion-exchange chromatography or a combination of the two techniques.

The gene encoding the desired immunogenic protein or polypeptide can be complexed to the carrier by combining these two components in solution containing NaCl at a concentration of 0.15 to 2 M. The carrier is added to the DNA and if a higher NaCl concentration is used, the solution of complex is dialyzed to reduce the salt concentration. In a preferred method, the carrier and DNA are both in 0.15 M NaCl and the carrier is added to the DNA to form a complex directly.

The gene encoding the desired immunogenic protein or polypeptide can be complexed to the carrier by a stepwise dialysis procedure. In a preferred method, for use with carriers made of polycations such as polylysine, the dialysis procedure begins with a 2 M NaCl dialyzate and ends with a 0.15 M NaCl solution. See e.g., Wu, G.Y. and Wu, C.H. J. Biol. Chem. (1987) 262:4429-4432. The gradually decreasing NaCl concentration results in binding of the DNA to the carrier.

The molecular complex can contain more than one copy of the same gene or one or more different genes. Preferably, the mass ratio of carrier to polynucleotide is about 1:5 to 5:1, preferably about 2:1. This ratio will vary considerably depending upon the components of the complex. For example, a conjugate with a lower proportion of polycation will typically be used at a higher ratio to the DNA.

The molecular complex of this invention can be administered parenterally. Preferably, it is injected intravenously. The complex is administered in solution in a physiologically acceptable vehicle.

Cells can be transfected in vivo for transient expression and secretion of the gene-encoded product. For prolonged expression and secretion, the gene can be administered repeatedly. Alternatively, the transfected target cell can be stimulated to replicate by surgical or pharmacological means to prolong expression of the incorporated gene. See, for example, U.S. Patent Application Serial No. 588,013, filed September 25, 1990, the teachings of which are incorporated by reference herein.

The molecular complex of this invention is adaptable for delivery of a wide range of genes to a specific cell or tissue. Preferably, the complex is targeted to the liver by exploiting the hepatic asialoglycoprotein receptor system which allows for in vivo transfection of hepatocytes by the process of receptor-mediated endocytosis. The liver has the highest rate of protein synthesis per gram of tissue. Thus, the molecular complex of this invention can be used to specifically target the liver as a site for high efficiency production of an immunogenic protein or polypeptide to thereby elicit an immune response to the protein or polypeptide.

The immunogen is typically, but not necessarily, a protein or polypeptide foreign to the host. It can be any protein or polypeptide which contains an epitope or epitopes (B or T cell), which are immunogenic in a host organism. For vaccination, the immunogen can be an immunogenic component or components of a pathogen, such as a virus, bacterium, or parasite, which can elicit a protective immune response against the pathogen. For example, the immunogen can be an envelope protein of a virus (e.g., HIV glycoprotein, HBV surface antigen) or a cell wall constituent of a bacterium.

In some cases, the protein or polypeptide against which the immune response is desired may be nonimmunogenic or poorly immunogenic. Such proteins or polypeptides can be coupled to an immunogenic carrier protein. This can be accomplished genetically by preparing a chimeric gene that encodes a fusion of the protein or polypeptide and the carrier.

The immunogen can be any agent against which antibodies are desired for diagnostic or therapeutic purposes. For example, the immunogen can be a component of a pathogen which elicits antibodies and/or cell useful for passive immunization against the pathogen. The immunogen can be a cell surface structure associated with a diseased cell such as tumor-associated antigen which elicits anti-tumor antibodies for diagnosis or therapy of tumor. The immunogen can also be any agent against which antibodies are needed for diagnostic assays. Examples include pathogens, hormones, cytokines, metabolites or drugs.

The method of this invention can be used to vaccinate an organism (human or other animal) to provide protection against infection. In one embodiment, a gene encoding an immunogenic protein or polypeptide which is a component of a pathogen is complexed to a conjugate of an asialoglycoprotein and a polycation. The resulting soluble complex is administered to a host organism to target liver cells in amounts sufficient to selectively transfect the cells and to provide sufficient production and secretion of the immunogen to elicit a protective immune response against the immunogen in the organism.

The method of this invention can also be used to produce polyclonal or monoclonal antibodies. For production of a polyclonal antibody, a gene encoding the immunogenic protein or polypeptide complexed with a carrier is administered to an organism (e.g., mouse, rabbit or goat) to elicit an immune response (antibody and/or cellular) against the immunogenic

protein or polypeptide. Antiserum which is specific for the immunogen is obtained from the immunized organism by known techniques.

For production of monoclonal antibodies, spleen cells or other antibody-producing cells are obtained from an organism immunized with the molecular complex. These cells are fused with appropriate immortalizing cells, such as myelomas, to produce hybridomas. The hybridomas are screened and those which produce antibodies specific for the immunogenic protein or polypeptide are selected.

In vivo gene transfer of an immunogenic protein or polypeptide to immunize an organism has several advantages over injection of a natural or recombinant protein which has been partially or completely purified to homogenity. Expression and secretion of an immunogenic protein or polypeptide in vivo eliminates the necessity of purifying an immunogen and can invoke a polyclonal antibody response that will be monospecific for the immunogenic protein or polypeptide. In addition, continuous expression and secretion of the immunogenic protein or polypeptide can boost the initial immune response in an organism.

This invention is illustrated by the following exemplification.

Exemplification

General

Polylysines (PL), obtained as the hydrobromide salts, and 3'dimethylaminopropyl ethyl carbodiimide (EDC) were from Sigma Chemical Co. Acrodisc syringe filters were obtained from Gelman Sciences.

Orosomucoid was isolated from pooled human plasma as described by Whitehead and Sammons (Biochim. Biophys. Acta (1966) 124:209-211) and desialylated to form asialoorosomucoid (ASOR) by treatment with 0.1 N H_2SO_4 at 80°C for 1 hour (Schmid et al. (1967) Biochem. J. 104:361-368). ASOR concentrations were determined using an OD_{280} of 0.92 for a 1.0 mg/mL solution in water. DNA concentrations were determined using an OD_{260} of 1.0 for a 50 µg/mL solution in water. HPLC purification of ASOR-PL conjugates was performed using a Brownlee Aquapore CX-300 cation exchange column (10 mm x 25 cm) obtained from Rainin Instrument Co. Dialysis tubing was obtained from Spectrum Medical Industries.

Synthesis of asialoorosomucoid-polylysine (ASOR-PL) conjugate

ASOR (60 mg/8 mL), PolyLysine HBr (5200 MW, 92 mg/3 mL) and EDC (42 mg/2 mL) were each dissolved in water. Each solution was filtered (0.45 µm) and the filter was washed with 2, 1 and 1 mL of water, respectively, which was combined with the corresponding solution. The pH's of the ASOR and the Polylysine solutions were adjusted to 7.4 with 132 µL and 420 µL of 0.1 N NaOH respectively. The EDC solution plus a 1 mL rinse was added to the ASOR solution with stirring and the pH of the mixture adjusted to 7.4 with 4 µL of 0.1 N NaOH. The polylysine solution plus a 1 mL rinse with water was added with stirring to the ASOR-EDC solution and the pH adjusted to 7.4 with 92 µL of 0.1 N NaOH. The reaction mixture was incubated for 24 hours at 37°C. The reaction mixture was then placed in 12,000-14,000

MW cut-off dialysis tubing and dialyzed against 20L of water for 24 hours and then twice against 8L of water for 3 hours. The resulting solution was lyophilized to yield 58 mg of ASOR-PL conjugate (38.0% based on ASOR and polylysine combined masses). Based on the OD_{280} of a 1.0 mg/mL solution in water (0.68) this conjugate was determined to be 74% ASOR by weight.

Alternative procedures

ASOR-PL conjugates prepared with polylysines of different molecular weights (Mr = 21,000 and 69,000) have also produced DNA complexes that produce similar positive results in animal experiments. These conjugates are synthesized in essentially the same manner as the conjugates made with 5.2Kd polylysine, with the additional step after dialysis of purification by HPLC followed by dialysis and lyophilization. The HPLC purification is performed on a Brownlee Aquapore cation exchange column (10 mm x 25 cm) with a flow rate of 4.5 mL/min. The column was eluted with buffers containing 0.1 M sodium acetate adjusted to acidic pH with HCl. A gradient from pH 5.0 to 2.0 was used to elute unconjugated ASOR, conjugate and polylysine. Conjugates typically elute at pH 2.5 to 2.0. In some runs, conjugate and excess polylysine overlap. In those instances, the front portion of the main peak that is rich in ASOR, as determined by the higher ratio of the absorbances at 280 nm and 230 nm, is collected and used for DNA complex formation as described above.

Gel retardation assays

A plasmid containing a head-to-tail dimer of the complete hepatitis B viral genome citation was made up to 100 µg/mL in 0.3 M NaCl and filtered (0.2 µm). An aliquot of the DNA solution (5 µL, 500 ng DNA) was placed in each of sixteen 1.5 mL polypropylene microtubes. A 1 mg/mL stock of ASOR-PL (5.2Kd polylysine) conjugate in water was prepared and filtered (0.2 µm). Solutions of the conjugate diluted with water were added to the DNA samples to create conjugate to DNA ratios in the range from 0.2:1 to 3.0:1 (w/w). Water was added to each sample to bring the final DNA concentration to 50 µg/mL and the NaCl concentration to 0.15 M. The samples were vortexed briefly, centrifuged for 30 seconds at 14,000 rpm and incubated at room temperature for 1 hour. Loading buffer (3.3 µL of 40% sucrose, 0.25% bromophenol blue) was added to each sample which was then loaded on to a 1% agarose gel. The gel was prepared by dissolving 0.4 g of agarose in 40 mL of TPE buffer (90 mM TRIS-phosphate, 2 mM EDTA, pH 8.0), adding 0.5 µL of a 10 mg/mL solution of ethidium bromide. The gel was 8 x 6.5 x 0.7 cm, the running buffer used was TPE buffer and a constant 50 V was applied for 1.5 hours. Extent of retardation of DNA was then observed under UV light. Full retardation was taken as that ratio of conjugate to DNA that retained all of the DNA in the sample well of the gel.

DNA-Conjugate complex formation

To plasmid (870 µL of a 0.2 µm-filtered solution at 915 µg/mL) was added water (523 µL) and 4.0 M NaCl to give a final DNA concentration of 500 µg/mL and a final NaCl concentration of 0.15-0.5 M. This sample was placed in a 5 mL vial and stirred. To ASOR-PL (5.2Kd polylysine) conjugate (597 µL of a 0.2 µm filtered solution at 2.0 mg/mL) was added water (796 µL) and 4.0 M NaCl (199 µL) to give a final conjugate concentration of 750 µg/mL and a final NaCl concentration of 0.15-0.5 M. The conjugate was added to the DNA solution at 6.7 µL/min (0.4 mL/h) via a peristaltic pump. After complete addition the plasmid DNA/ASOR-PL complex was filtered (0.2 µm) and determined by UV to contain 250 µg/mL of DNA. Complexation was verified by running an agarose gel of plasmid DNA (500 ng) and the complex (500 ng of DNA).

In vivo transfection of animals and detection of HBsAg

BALB/c mice 8-16 weeks of age, purchased from Harlan Sprague Dawley, Inc. (Madison, WI), were injected intravenously with 34 µg to 125 µg of HBV DNA contained in a DNA/ASOR-PL complex. Fifteen - 30 minutes after injection, mice were anesthetized with ether and a 2/3 partial hepatectomy performed to induce a more prolonged state of gene expression. For this procedure a midline incision was made from just below the sternum extending midway down the abdomen. From the opening in the peritoneal cavity the liver was exposed and surgical silk was wrapped around the major bottom lobe of the liver. The silk was tied off just below the point where the hepatic

veins join the inferior vena cava. The ligated region of the liver was excised and the incision closed with wound clips.

Serum samples were collected from mice at varying intervals after injection of the DNA/ASOR-PL complex and initially tested for the presence of hepatitis B surface antigen in the circulation using an ELISA kit from Abbott Laboratories (Abbott Park, IL). This test can detect as little as 5 pico grams of antigen.

Enzyme-linked immunosorbant assay for detection of antibodies against HBsAg

Each well of an Immulon-4 96 well plate (Fisher Scientific) was coated with 500 ng/well of HBsAg (provided by Catherine Wu, University of Connecticut). For binding, HBsAg was suspended in 0.01 M sodium bicarbonate buffer, pH 9.6 and incubated for 60 minutes at 37°C. The wells were washed 5X with phosphate buffered saline containing 0.025% Tween 20 (PBS-Tween). Blocking of unreacted sites was accomplished by adding to each well 200 µl of 2.5% bovine serum albumin w/v (Sigma) suspended in PBS; incubation was carried out at 37°C for 1-2 hours. The wells were again washed 5X with PBS-Tween. Sera from each of the animals was initially diluted 1:3 in PBS and 100 µl added to each well. To each of the wells in the vertical row the serum was diluted 3 fold in successive wells. Serum samples and dilutions were incubated for 1 hour at 37°C. Each well was again washed 5X with PBS-Tween. To each well 100 µl of a 1:1000 dilution of goat antibody against mouse IgG heavy and light chain,

labeled with horse radish peroxidase (HRP), was added and incubated for 1 hour at 37°C. The wells were again washed 5X with PBS-Tween. Finally, to each well 100 µl of tetramethylbenzidene (TMB) peroxidase substrate (Kirkegard and Perry, Gaithersburg, MD) was added and blue color was allowed to develop for 15-30 minutes. The development of the catalyzed color reaction was evaluated spectrophotometrically with a plate reader.

Results

Pre-injection serum samples tested negative for HBsAg. Following intravenous injection of the DNA/ASOR-PL complex, sera from animals were tested for the presence of HBsAg. In 8 out of 10 animals antigen was detected in the sera of animals as early as 3 days or as late as 7 days after injection. All animals which tested positive for the presence of HBsAg became negative for HBsAg 7-10 days after first detection antigen. Sera from these animals were then tested for the presence of antibodies against HBsAg. The antibody titers are presented in Figure 1 as the reciprocal dilution of the sera which was above background levels detected by the plate reader. The results in Figure 1 show that 8 out of 10 animals developed antibodies against HBsAg ranging in relative antibody titer from about 250 to 700. Animal #3 showed an antibody titer of about 50 and in animal #1 no HBsAg or antibody was detected. This data shows that an immune response can be evoked by method of this invention.

It should be noted that animal #3 tested on two separate occasions (days 4 and 10 after injection of DNA/ASOR-PL) remained negative for HBsAg, but developed antibodies against HBsAg. The ELISA test for HBsAg has a sensitivity of about 5 pg. Typically, larger amounts of exogenously administered immunogens are required to evoke an immune response in an organism. This suggests that the introduction of a foreign protein via endogenous production in host cells may be a more efficient process for evoking an immune response than by injecting foreign protein directly into animals.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

Claims

1. A soluble molecular complex for targeting a gene encoding an immunogenic protein or polypeptide to a specific cell, the complex comprising nucleic acid containing an expressible gene encoding the immunogenic protein or polypeptide complexed with a carrier of a cell-specific binding agent and a gene-binding agent.

2. A soluble molecular complex of claim 1, wherein the expressible gene is DNA.

3. A soluble molecular complex of claim 1, wherein the immunogenic protein or polypeptide is a component of a pathogen.

4. A soluble molecular complex of claim 1, wherein the gene-binding agent is a polycation.

5. A soluble molecular complex of claim 4, wherein the polycation is polylysine.

6. A soluble molecular complex of claim 1, wherein the cell-specific binding agent binds a surface receptor of the cell which mediates endocytosis.

7. A soluble molecular complex of claim 6, wherein the cell-specific binding agent is a ligand for an asialoglycoprotein receptor.

8. A soluble molecular complex of claim 7, wherein the ligand is an asialoglycoprotein and the targeted cell is a hepatocyte.

9. A soluble molecular complex of claim 1, wherein the expressible gene is complexed with the gene-binding agent by a noncovalent bond.

10. A soluble molecular complex of claim 1, wherein the cell-specific binding agent is linked to the gene-binding agent by a covalent bond.

11. A soluble molecular complex of claim 1, wherein the expressible gene is complexed with the gene-binding agent so that the gene is released in functional form under intracellular conditions.

12. A therapeutic composition comprising a solution of the molecular complex of claim 1 and a physiologically acceptable vehicle.

13. A soluble molecular complex for targeting a gene encoding an immunogenic protein or polypeptide to a hepatocyte, the complex comprising an expressible gene encoding the immunogenic protein or polypeptide complexed with a carrier of a ligand for the asialoglycoprotein receptor and a polycation.

14. A soluble molecular complex of claim 13, wherein the immunogenic protein or polypeptide is a component of a pathogen.

15. A soluble molecular complex of claim 14, wherein the immunogenic protein or polypeptide is hepatitis B surface antigen.

16. A soluble molecular complex of claim 13, wherein the polycation is polylysine.

17. A soluble molecular complex of claim 13, wherein the gene is contained in an expression vector along with genetic regulatory elements necessary for expression of the gene by the hepatocyte.

18. A soluble molecular complex of claim 17, wherein the expression vector is a plasmid or viral DNA.

19. A method of immunization comprising administering to an organism a soluble molecular complex comprising an expressible gene encoding an immunogenic protein or polypeptide complexed with a carrier of a cell-specific binding agent and a gene-binding agent, in an amount sufficient to elicit an immune response against the immunogenic protein or polypeptide in the organism.

20. A method of claim 19, wherein the expressible gene is DNA.

21. A method of claim 19, wherein the immunogenic protein or polypeptide is a component of a pathogen.

22. A method of claim 19, wherein the gene-binding agent is a polycation.

23. A method of claim 21, wherein the polycation is polylysine.

24. A method of claim 19, wherein the cell-specific binding agent binds a surface receptor of the cell which mediates endocytosis.

25. A method of claim 24, wherein the cell-specific binding agent is a ligand for an asialoglycoprotein receptor.

26. A method of claim 25, wherein the ligand is an asialoglycoprotein and the targeted cell is a hepatocyte.

27. A method of claim 19, wherein the molecular complex is administered intravenously.

28. A method of producing polyclonal antibodies, comprising:
a) administering to an organism a soluble molecular complex comprising an expressible gene encoding an immunogenic protein or polypeptide complexed with a carrier of a ligand for the asialoglycoprotein receptor and a polycation, in an amount sufficient to elicit an immune response against the immunogenic protein or polypeptide in the organism; and
b) obtaining antisera from the organism.

29. A method of claim 28, wherein the immunogenic protein or polypeptide is a component of a pathogen.

30. A method of claim 28, wherein the polycation is polylysine.

31. A method of producing monoclonal antibodies, comprising:
a) administering to an organism a soluble molecular complex comprising an expressible gene encoding an immunogenic protein or polypeptide complexed with a carrier of a ligand for the asialoglycoprotein receptor and a polycation, in an amount sufficient to elicit an immune response against the immunogenic protein or polypeptide in the organism;

b) obtaining antibody-producing cells from the organism;

c) fusing the antibody-producing cells with immortalizing cells to produce hybridoma cells; and

d) selecting hybridomas which produce antibodies specific for the immunogenic protein or polypeptide.

32. A method of claim 31, wherein the immunogenic protein or polypeptide is a component of a pathogen.

33. A method of claim 31, wherein the polycation is polylysine.

34. Use of a soluble molecular complex according to claim 1 for the manufacture of a medicament for use in immunization against the immunogenic protein or polypeptide.

35. Use according to claim 34, wherein the expressible gene is DNA.

36. Use according to claim 34, wherein the immunogenic protein or polypeptide is a component of a pathogen.

37. Use according to claim 36, wherein the immunogenic protein or polypeptide is hepatitis B surface antigen.

38. Use according to claim 34, wherein the gene-binding agent is a polycation.

39. Use according to claim 37, wherein the polycation is polylysine.

40. Use according to claim 34, wherein the cell-specific binding agent binds a surface receptor of the cell which mediates endocytosis.

41. Use according to claim 40, wherein the cell-specific binding agent is a ligand for an asialoglycoprotein receptor.

42. Use according to claim 41, wherein the ligand is an asialoglycoprotein and the targeted cell is a hepatocyte.

43. Use according to claim 34, wherein the molecular complex is administered intravenously.

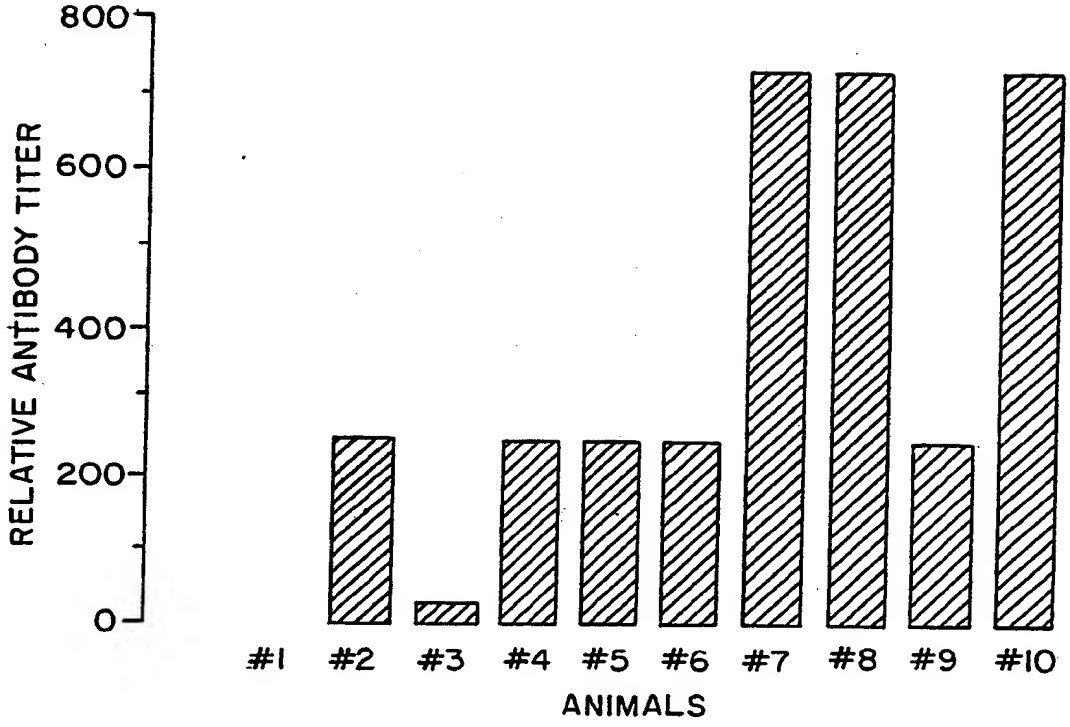


FIG. 1

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 47/48 — A3

(11) International Publication Number: WO 92/20316

(43) International Publication Date: 26 November 1992 (26.11.92)

(21) International Application Number: PCT/US92/03875

(22) International Filing Date: 14 May 1992 (14.05.92)

(30) Priority data:
699,891 14 May 1991 (14.05.91) US

(71) Applicants: UNIVERSITY OF CONNECTICUT [US/US]; Storrs, CT 06269 (US). TARGETECH, INC. [US/US]; 290 Pratt Street, Meriden, CT 06450 (US).

(72) Inventors: WU, George, Y. ; 15 Rundelane, Bloomfield, CT 06002 (US). SPITALNY, George, L. ; 6 Brookfield Court, Cheshire, CT 06410 (US). FINDEIS, Mark, A. ; 234 Sherman Avenue, Apt. #29, Meriden, CT 06450 (US).

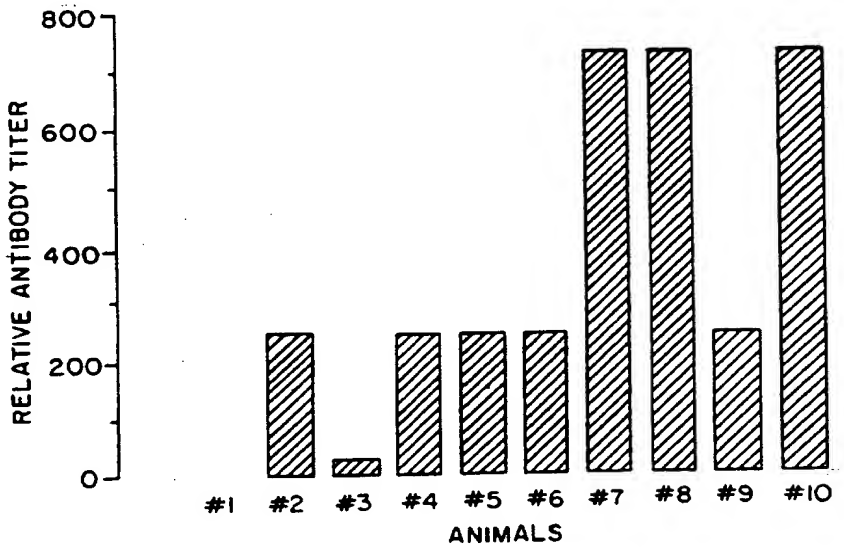
(74) Agents: DECONTI, Giulio, A., Jr. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
21 January 1993 (21.01.93)

(54) Title: TARGETED DELIVERY OF GENES ENCODING IMMUNOGENIC PROTEINS



(57) Abstract

Molecular complexes can be used to target a gene encoding an immunogenic protein or polypeptide to a specific cell *in vivo*. The gene is incorporated into the target cell, expressed and the gene-encoded product is secreted and an immune response against the immunogenic protein or polypeptide is elicited. The molecular complex comprises an expressible gene encoding a desired immunogenic protein or polypeptide complexed with a carrier of a cell-specific binding agent and a gene-binding agent. The cell-specific binding agent is specific for a cellular surface structure which mediates internalization of ligands by endocytosis. An example is the asialoglycoprotein receptor of hepatocytes. The gene-binding agent is a compound such as a polycation which stably complexes the gene under extracellular conditions and releases the gene under intracellular conditions so that it can function within a cell. The molecular complex is stable and soluble in physiological fluids and can be used to elicit an immune response against a variety of immunogens in an organism for the purpose of vaccination or for the production of antibodies.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FI**	Finland	**ML**	Mali
AU	Australia	**FR**	France	**MN**	Mongolia
BB	Barbados	**GA**	Gabon	**MR**	Mauritania
BE	Belgium	**GB**	United Kingdom	**MW**	Malawi
BF	Burkina Faso	**GN**	Guinea	**NL**	Netherlands
BG	Bulgaria	**GR**	Greece	**NO**	Norway
BJ	Benin	**HU**	Hungary	**PL**	Poland
BR	Brazil	**IE**	Ireland	**RO**	Romania
CA	Canada	**IT**	Italy	**RU**	Russian Federation
CF	Central African Republic	**JP**	Japan	**SD**	Sudan
CG	Congo	**KP**	Democratic People's Republic of Korea	**SE**	Sweden
CH	Switzerland			**SN**	Senegal
CI	Côte d'Ivoire	**KR**	Republic of Korea	**SU**	Soviet Union
CM	Cameroon	**LI**	Liechtenstein	**TD**	Chad
CS	Czechoslovakia	**LK**	Sri Lanka	**TG**	Togo
DE	Germany	**LU**	Luxembourg	**US**	United States of America
DK	Denmark	**MC**	Monaco		
ES	Spain	**MG**	Madagascar		

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/03875

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K47/48

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Search.[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 264, no. 29, 15 October 1989, BALTIMORE,USA pages 16985 - 16987 WU ET AL 'TARGETING GENES:DELIVERY AND PERSISTENT EXPRESSION OF A FOREIGN GENE DRIVEN BY MAMMALIAN REGULATORY ELEMENTS IN VIVO' see the whole document ---	1-43
A	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 266, no. 6, 25 February 1991, BALTIMORE,USA pages 3361 - 3364 KATO ET AL 'EXPRESSION OF HEPATITIS B VIRUS SURFACE ANTIGEN IN ADULT RAT LIVER' see the whole document --- -/--	1-43

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29 OCTOBER 1992	24. 11. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	SITCH W.D.C.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0 161 188 (MERCK AND CO INC.) 13 November 1985 see claims 1-40	1-43

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/03875

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
ALTHOUGH CLAIMS 19-33 ARE DIRECTED TO A METHOD OF TREATMENT OF THE HUMAN/ANIMAL BODY THE SEARCH HAS BEEN CARRIED OUT AND BASED ON THE ALLEGED EFFECTS OF THE COMPOUND/COMPOSITION.

2. [X] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
THE TERMS USED IN CLAIM 1 AND RELATED CLAIMS SUCH AS "IMMUNOGENIC PROTEIN OR POLYPEPTIDE"; "CARNIER OF A CELL-SPECIFIC BINDING AGENT" ARE NOT SUFFICIENTLY WELL DEFINED TO CONFORM WITH THE REQUIREMENTS OF ART.6 PCT. THESE TERMS HAVE THUS BEEN SEARCHED IN THE LIGHT OF THE NAMED EXAMPLES OF SUCH GIVEN IN THE EXAMPLES OF THE APPLICATION.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9203875 SA 61004

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 29/10/92

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0161188	13-11-85	AU-B-	589559	19-10-89
		AU-A-	4221485	14-11-85
		CA-A-	1259450	12-09-89
		JP-A-	60248622	09-12-85
		US-A-	4882317	21-11-89
		US-A-	4695624	22-09-87